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SUMMARY

The pag gene, which codes for protective antigen (PA), a common component of the lethal and edema toxins of Bacillus anthracis, was cloned and express in Escherichia coli. Nested deletions of pag were generated into the C-terminus coding region. REcombinant proteins were analyzed by Western blot with either an anti-PA polyclonal antisera or two monoclonal antibodies that neutralized lethal toxin and edema toxin activities by inhibiting the binding of PA to cell receptors. Localization of the receptor binding domain within the C-terminal region of PA was suggested by the inability of the monoclonal antibodies 3B6 and 14B7 to recognize the recombinant proteins expressed by C-terminal deletions of the pag gene.

LOCATION OF RECEPTOR-BINDING REGION OF PROTECTIVE ANTIGEN FROM *BACILLUS ANTHRACIS*

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Summary: The *pag* gene, which codes for protective antigen (PA), a common component of the lethal and edema toxins of *Bacillus anthracis*, was cloned and expressed in *Escherichia coli*. Nested deletions of *pag* were generated into the C-terminus coding region. Recombinant proteins were analyzed by Western blot with either an anti-PA polyclonal antisera or two monoclonal antibodies that neutralized lethal toxin and edema toxin activities by inhibiting the binding of PA to cell receptors. Localization of the receptor binding domain within the C-terminal region of PA was suggested by the inability of the monoclonal antibodies 3B6 and 14B7 to recognize the recombinant proteins expressed by C-terminal deletions of the *pag* gene.

The exotoxin produced by *Bacillus anthracis* strains containing plasmid pXO1 is a combination of two toxins, lethal toxin and edema toxin. Lethal toxin is composed of lethal factor (LF) and protective antigen (PA), causes death in certain experimental animals (1), and lyses certain mouse macrophages as well as a mouse macrophage-like cell line (2,3). Edema toxin, which produces edema in the skin of experimental animals (4), is composed of edema factor (EF) and PA. Edema factor is a calcium- and calmodulin-dependent adenylate cyclase (5). Both lethal toxin and edema toxin conform to the A/B toxin model, where PA acts as the B-receptor binding subunit for both LF and EF A-subunits.

The current model (6,7) of toxin activity for both toxins suggests that PA (83,000 Da) undergoes proteolytic cleavage near residues 164-167 after binding to a cell receptor. This cleavage releases a 20,000-Da fragment from the N-terminal end of PA. The 63,000-Da, membrane-bound fragment is capable of binding either LF

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or EF. Toxic activity occurs after internalization by receptor-mediated endocytosis (2).

Molecular cloning of the PA and EF genes into the E. coli vector pBR322 plasmid was accomplished by Vodkin et al. (8), and Robertson et al. cloned the LF gene into the E. coli vector PUC8 (9).

In a recent report, two monoclonal antibodies (Mab; 3B6 and 14B7), directed against PA, neutralized lethal toxin and edema toxin activities by inhibiting the binding of ^{125}I-PA to its cognate cell receptor (10). In this paper, we localized the receptor-binding region of PA to its C-terminal region, based upon Western blot analysis by using MAbs 3B6 and 14B7 against recombinant proteins expressed by E. coli containing deletions in the PA gene.

Methods

Subcloning of pag into pBluescript vector and generation of pag deletions. The pag gene, contained within the E. coli plasmid pPA26 (11), was subcloned into the polylinker region of the phagemid vector pBluescript KS M13+ by subjecting both plasmids to cleavage by the restriction endonucleases BamHI and ClaI. After dephosphorylation of the vector DNA with bacterial alkaline phosphatase, it was ligated to the appropriate gel-purified DNA fragment from pPA26 by T4 DNA ligase (Figure 1). E. coli strain JM-109 competent cells were transformed with the ligated DNA and plated on yeast extract/tryptone agar containing ampicillin (100 μg/ml). Plasmid DNA containing the PA gene, pBLKSPPA, was isolated by standard techniques (12). Nested DNA deletions into the C-terminus coding region of the PA gene were generated by incubating the pBLKSPPA with two enzymes, BamHI and BstXI, which created one DNA end that was resistant to digestion by exonuclease III (13). The single-stranded end was blunted with mung bean nuclease and a blunt-end ligation was performed before transformation of competent JM-109 cells, as previously described. Colonies were picked and streaked on solid media containing ampicillin. After overnight growth, plasmid DNA was extracted from bacterial colonies collected from each streak by using a rapid isolation and screening protocol (14). Estimations of plasmid size were made by comparing DNA migration distances in agarose gel electropherograms with DNA migrations of a supercoiled plasmid standard. DNA was visualized in the agarose gels by staining with ethidium bromide.

Sequence analysis of pBKPPAΔ65 deletion clone. Chain termination sequencing reactions (15) were performed on alkali-denatured plasmids with fluorescent-dye-labelled reverse sequencing primers

and dideoxynucleotide inhibitors (16) to terminate specifically the elongation reactions catalyzed by T7 DNA polymerase (17). Labelled DNA fragments were electrophoretically fractionated in a 6% polyacrylamide gel. DNA sequences were detected and analyzed with an automated DNA sequencer (Applied Biosystems, Inc., 18). Predicted protein size was based on a comparison of the length of the protein expressed from the deleted gene region with that deduced from the total sequence of the PA gene (11).

Expression and Extraction of Recombinant PA. Cultures (10 ml medium; 2XYT broth, 0.3% glucose, and 100 μg ampicillin per ml) were started from a single recombinant E. coli colony isolated from plates containing ampicillin. After incubation at 37°C in a shaking incubator overnight, 100 μl was inoculated into 30 ml of fresh medium. Chloramphenicol (170 μg/ml) was added to the cultures when growth reached a cell density of ca. 1.0 at A_{600} and incubation continued overnight (19, 20). Cells were pelleted, washed with 2XYT broth, and incubated for 1 h in 30 ml of fresh medium. The cells were harvested, resuspended in 2 ml of 0.125 M Tris/HCl, pH 6.8, 1 mg lysozyme per ml, 1 mM phenylmethylsolfonyl fluoride (PMSF) and 50 mM EDTA, and incubated for 60 min at room temperature.

SDS-PAGE and Western blots. Samples were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels under reducing conditions (21) after addition of 1 ml 3x SDS sample buffer to the cells described above. The separated proteins were electrophoretically transferred to nitrocellulose paper (22) and membrane-bound proteins were incubated with either rabbit anti-PA antiserum or MAb 3B6 or 14B7, followed by horseradish peroxidase conjugated to goat anti-rabbit antiserum or goat anti-mouse antiserum, respectively. Components were visualized by 4-chloro-1-naphthol substrate.

Results and Discussion

Expression of the pag gene in E. coli has been nominal (personal observations). An increase in the amount of protein expressed by the recombinant E. coli clones was obtained by using chloramphenicol to amplify the number of recombinant plasmids per cell before harvesting (19, 20).

A schematic diagram depicting the relative size of the pag gene within the pBluescript plasmid for the parent clone (pBLKSPPA) and relative positions of the nested deletions into the C-terminus coding region of pag for the deletion clones (pBKPPAΔ70, pBKPPAΔ69,

pBKPPAΔ65) described in this report are shown in Figure 2. The estimated size of the pBluescript plasmid clone containing the B. anthracis DNA excised from pPA26 is indicated in the right margin. The two B. anthracis coding regions include pag and the uncharacterized ORF1 (open reading frame).

PA protein expressed by these recombinant clones containing the pag gene or deletions of the C-terminus coding region of pag were identified by Western blot with either rabbit anti-PA antiserum (Figure 3A) or MAb 3B6 (Figure 3B). Recombinant mature PA was expressed as two bands on SDS-PAGE gels. The larger molecular weight protein is believed to represent incomplete cleavage of the Bacillus signal sequence from the final product by the E. coli signal peptidases. Monoclonal antibody 3B6 recognized the parent clone, pBLKSSPA, and two deletion clones, pBKPPAΔ70 (7.0 kb) and pBKPPAΔ69 (6.9 kb), but did not recognize deletion clone pBKPPAΔ65 (6.5 kb) (Figure 3B). Similar results were observed with MAb 14B7 (data not shown). Both of these Mabs have been shown to block the binding of PA to its target cell-receptor (10). Based upon SDS-PAGE gels, pBKPPAΔ70 was nearly full-length mature PA and pBKPPAΔ69 lacked about 30 amino acid residues of mature PA. Molecular weights of PA recombinant proteins estimated from the SDS-PAGE gels are shown in Table 1. The molecular weight of pBKPPAΔ65 was determined from results of end terminal sequencing, which identified a deletion of 155 amino acids from the C-terminal end of mature PA. This was consistent with the finding of a major protein of 72,000 daltons observed on Western blot which reacted with rabbit anti-PA serum (Figure 3A, lane 6) but not with MAb 3B6 (Figure 3B, lane 6). DNA sequencing also identified seven nucleotides from the vector region of the plasmid which were in-frame with the C-terminus coding region of the deleted PA gene.

These results strongly suggest that the MAbs 3B6 and 14B7 react with the C-terminal region of PA. Since both MAb 3B6 and 14B7 have previously been demonstrated to block binding of PA to its cell receptor, this further implies that the cell receptor binding domain of PA is located at the C-terminal end of the

molecule. Further work is required to determine which specific residues within the 155 amino acids at the C-terminal region of PA are recognized by the MAbs and the receptor.

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TABLE 1. Plasmid size and recombinant protein molecular mass from PA recombinant E. coli clones

Recombinant Clone	Plasmid size (kb)[a]	Molecular Mass (Da)[b]
pBLKSPPA	7.2	83,000 & 85,000
pBKPPAΔ70	7.0	83,000 & 85,000
pBKPPAΔ69	6.9	81,000 & 83,000
pBKPPAΔ65	6.5	72,000

(a) Plasmid sizes were estimated from agarose gels.
(b) Molecular masses were estimated from SDS-PAGE gels except for pBKPPAΔ65, which was calculated from plasmid DNA sequence data.

Figure 1. Molecular Cloning of B. anthracis pag Locus into pBluescript KS M13+ Phagemid Vector. The light region in pPA26 represents 4.0 kilobases (kb) of DNA from the E. coli plasmid pBR322 outside the unique BamHI and ClaI restriction endonuclease recognition sites. The dark region represents 4.3 kb of DNA from B. anthracis plasmid pXO1 which contained the pag locus. The stippled region represents the 2.9 kb of DNA from the E. coli phagemid pBluescript KS M13+ outside the unique BamHI and ClaI sites in the multiple cloning region. The vector DNA was dephosphorylated with bacterial alkaline phosphatase (BAP) prior to ligation to the gene region forming the 7.2-kb recombinant molecule, pBLKSPPA.

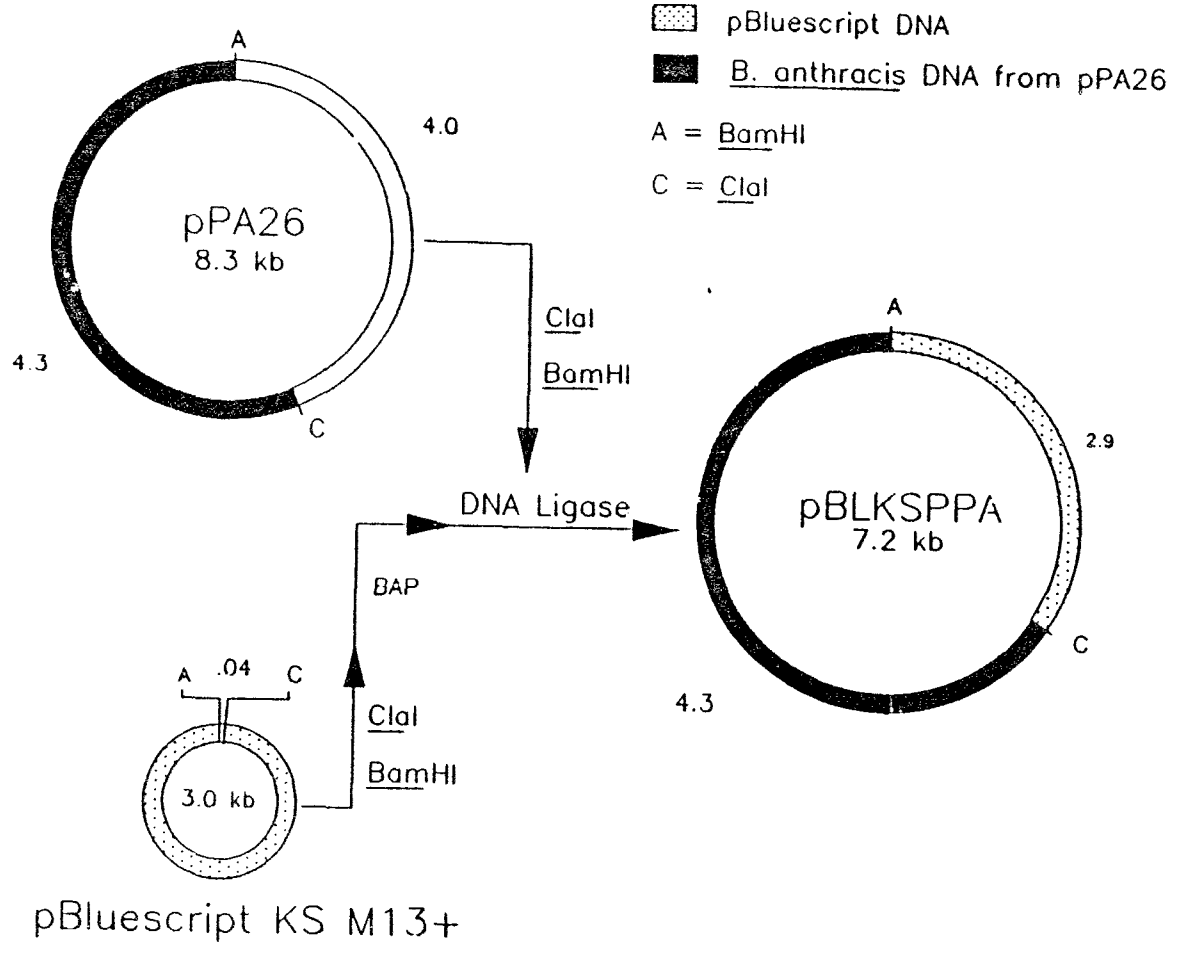

pBluescript DNA
B. anthracis DNA from pPA26
A = BamHI
C = ClaI
A
4.0
pPA26
8.3 kb
4.3
C
ClaI
BamHI
DNA Ligase
BAP
A
2.9
pBLKSPPA
7.2 kb
C
4.3
A .04 C
ClaI
BamHI
3.0 kb
pBluescript KS M13+

Figure 2. pBluescript Deletion Clones of Protective Antigen Gene. Nested sets of deletion clones into the C-terminal coding region of the pag locus were prepared by controlled exonuclease digestion of pBLKSPPA DNA doubly cut with BstXI to give a 3'-overhanging protected end and with BamHI. The orientation of the vector region from pBluescript KS M13+ is indicated with the T3 promoter priming site. The length and direction of transcription is indicated for pag and the uncharacterized open reading frame gene ORF1. The size of the phagemid generated is listed in the right margin while the clone descriptor is listed in the left margin. A, BamHI; B, BstXI; C, ClaI; K, KpnI; 3, T3 primer; ORF 1, open reading frame.

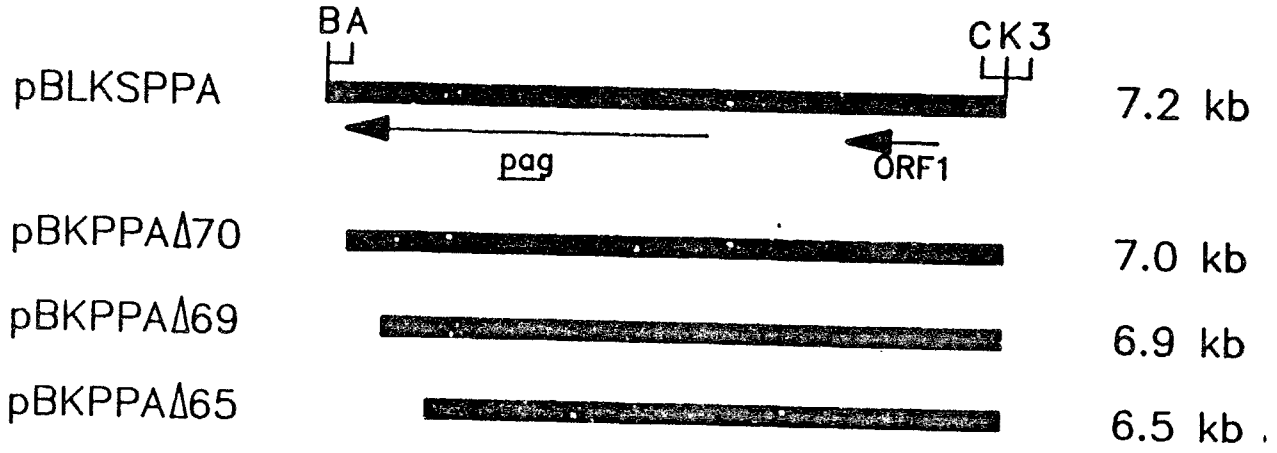
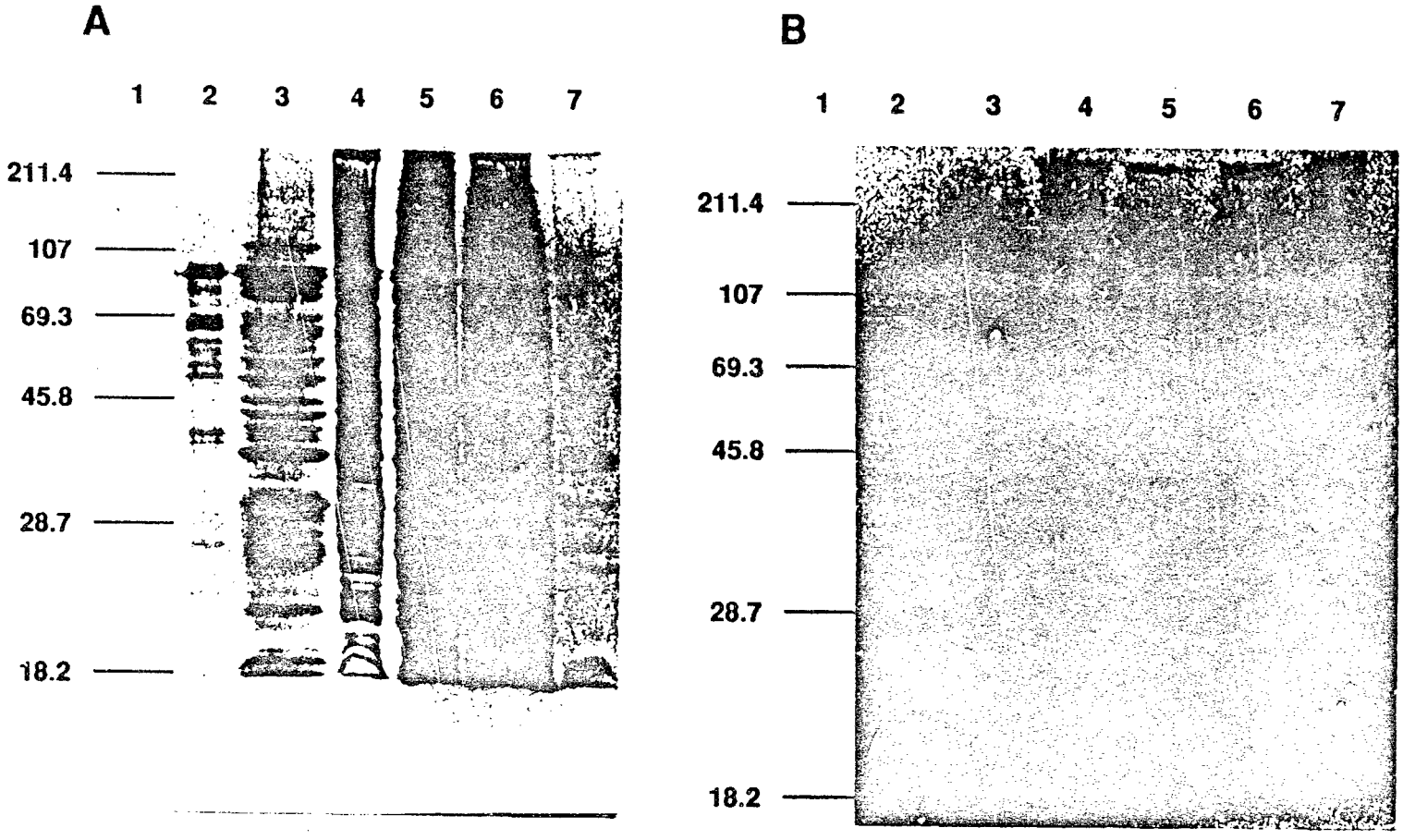

BA
CK3
pBLKSPPA
7.2 kb
pag
ORF1
pBKPPAΔ70
7.0 kb
pBKPPAΔ69
6.9 kb
pBKPPAΔ65
6.5 kb

Figure 3. Western blot analysis of PA recombinant proteins expressed in E. coli. (A) Western blot with polyclonal rabbit anti-PA antiserum: lane 1, molecular weight standards (in kilodaltons); lane 2, PA; lane 3, pBLKSPPA; lane 4, pBKPPAΔ70; lane 5, pBKPPAΔ69; lane 6, pBKPPAΔ65; lane 7, E. coli containing pBluescript plasmid. (B) Western blot with MAb 3B6 (8): lane 1, molecular weight standards (in kilodaltons); lane 2, PA; lane 3, pBLKSPPA; lane 4, pBKPPAΔ70; lane 5, pBKPPAΔ69; lane 6, pBKPPAΔ65; lane 7, E. coli containing pBluescript plasmid.


A
1 2 3 4 5 6 7
211.4
107
69.3
45.8
28.7
18.2
B
1 2 3 4 5 6 7
211.4
107
69.3
45.8
28.7
18.2

Abbreviations: Da, dalton; EDTA, ethylenediaminetetraacetic acid; EF, edema factor; kb, kilobase; LF, lethal factor; MAb, monoclonal antibody; PA, protective antigen; PAGE, polyacrylamide gel electrophoresis.

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